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(54) A method for determining the Histocompatibility locus antigen class II

(57) The present invention relates to methods and materials for determining the HLA Class II type of a subject, wherein group-specific sequences are used to design primer molecules which may be used in amplification protocols which accurately identify the HLA group(s) and/or allele(s) carried by the subject.

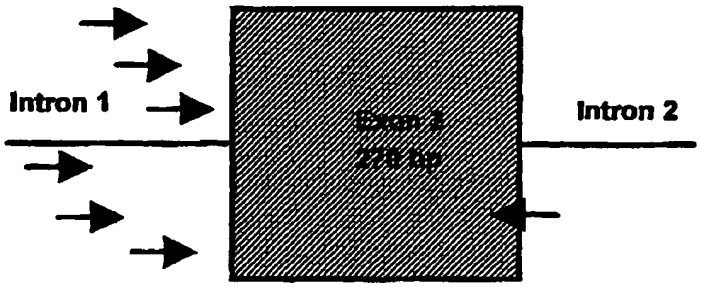

Fig. 1

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Description

The present invention relates to methods and materials for determining the HLA Class II type of a subject, wherein group-specific sequences are used to design primer molecules which may be used in amplification protocols which accurately identify the HLA group(s) and/or allele(s) carried by the subject.

The Human Leukocyte Antigen ("HLA") Class II complex comprises the genes coding for HLA-DR, HLA-DQ and HLA-DP. The complex comprises several gene loci among which the following are especially to be mentioned: DRB1, DRB2, DRB3, DRB4, DRB5, DRB6, DRB7, DRB8, DRB9, DQA1, DQA2, DQB1, DQB2, DPA1, DPA2, DPB1, DPB2.

The HLA Class II genes are highly polymorphic among individuals. This variability is of particular relevance when tissue or organ transplantation between a donor and a host is contemplated. The histocompatibility antigens of donor and host should be as similar as possible to avoid both immune rejection of the transplanted tissue as well as graft-versus-host disease. It is therefore important to accurately identify the HLA types of donor and host. In view of the exigencies implicit in tissue transplantation, it is desirable that the typing be accomplished as efficiently as possible.

Methods for determining alleles of HLA in a patient sample have been heavily investigated because of the functional importance of these genes in transplant tissue matching and autoimmune diseases. The first tests developed used immunological methods to identify epitopes expressed by various HLA loci. These tests (*e.g.*, the complement-dependent cytotoxicity assay described in Terasaki and McClelland, Nature, 204:998, (1964)) identified broad serological specificities but were not capable of distinguishing between allelic members of a group, and sometimes misidentified groups altogether. Unfortunately, even the most accurate of such low resolution assays cannot detect and distinguish all functionally significant transplant antigens (Anasetti et al. Hum. Immunol., 29:70 (1990)).

High resolution tests performed at the nucleic acid level which distinguish among alleles of each group have become the focus of recent research. Current methods of high resolution typing include the following.

The Sequence Specific Oligonucleotide Probes ("SSOP") technique, as described in United States Patent No. 5,451,512 assigned to Hoffman-La Roche, Inc., uses a reverse dot blot format, wherein HLA-A probes are immobilized on a membrane, and the labelled target (patient sample) DNA is hybridized to the membrane-bound probe (as described in Saiki et al., 1989, Proc. Natl. Acad. Sci. 86:6230-6234). The pattern of hybridization to the probes on the dot-blot gives information regarding the HLA type of the individual. However, because hybridization is inherently not sufficiently specific to rule out minor differences in sequence between probe and patient sample, there is a possibility that the patient sample may contain an allelic variant which is not accounted for.

Another nucleic acid-based test is the Amplification Refractory Mutation System (ARMS) as described in the "HLA Class I SSP ARMS-PCR Typing Kit" Reference Manual, June 1995 edition, published by the Imperial Cancer Research Fund. This assay is based on the need for complementarity (matching) between the 3' end of an amplification primer and a target DNA sequence. Absent such matching, the primer will not function properly and no fragment will be amplified. Sequence information is deduced by determining, for various pairs of primers acting on target DNA from a patient sample, whether or not a fragment is successfully amplified. The accuracy of the technique is limited by the number of primer pairs tested and by the possibility that allelic variations exist in regions of DNA which lie between the primers.

In order to overcome the foregoing shortcomings, it has been proposed that typing be accomplished by direct DNA sequencing (Santamaria et al., "HLA Class II typing: direct sequencing of DRB, DQB, and DQA genes", Hum. Immunol. 33, 69-81 (1992); Santamaria et al., "HLA Class I Sequence-Based Typing" Hum. Immunol. 37, 39-50 (1993); WO 9219771; US Pat. 5,424,184). However, while direct sequencing of a patient's Class II HLA locus may conceptually be the most accurate, such sequencing may require a time-frame unsuitable for clinical practice. The success of direct sequencing methods may be expected to rely upon the design of efficient protocols and relevant primer sequences.

Prior to the present invention, direct sequencing protocols have exhibited a number of disadvantages. For example, the method of Santamaria et al., supra, fails to provide sufficient information because it focuses on cDNA (exon) sequences which, in view of exon sequence diversity, offer a very limited selection of conserved primer hybridization sites. In addition, because the Santamaria sequencing primers hybridize within an exon, they do not provide information for DNA sequence upstream of the primer which is potentially decisive for distinguishing among alleles. Further, the sites disclosed were determined before the recent discovery of dozens of more alleles that now need to be considered in identifying HLA type.

Intron sequences could provide the preferred hybridization sites for amplification and sequencing primers for the HLA Class II genes because they may provide the DNA sequence of the full exon.

The existence of introns, interrupting the coding regions of nuclear genes, is a typical feature of genomic DNA in complex eukaryotes. Introns are usually much longer than exons and are responsible for the major part of structural genes. In the present invention especially the sequences of intron 1 and intron 2 which are located at the 5'-end and 3'-end, respectively, of exon 2 are especially important.

A number of researchers have made limited use of intron based oligonucleotides for limited aspects of HLA Class I typing.

The European patent application EP-A-90.309107.2 of GENETYPE AG discloses an intron sequence analysis method for detection of adjacent and remote locus alleles as haplotypes. According to this method genomic DNA is amplified with a primer pair that spans a non-coding region sequence (intron). The used primer pair defines a DNA sequence which is in genetic linkage with an allele to be detected.

Blasczyk et al. (Tissue Antigens 1996: 47: 102-110) used exon based amplification primers to determine group specificity of HLA Class I. After amplification, universal sequencing primers located in intron 2 were used to sequence the amplified fragment. The paper does not disclose any intron sequence motifs from intron 1 or 3 or the 5' untranslated region.

Cereb et al. (Tissue Antigens 1995: 45:1-11), undertook the identification of intron sequences useful for locus-specific amplification primer sets for all Class I genes. These primer sets were designed to amplify all alleles of the same locus. No group specific amplification primers were sought or reported. Further, amplified fragments were characterized by SSOP and not by direct sequencing.

Li W.-H. et al. (Fundamentals of molecular evolution, Sunderland$_1$ MA; Sinauer Associates (1991)] has speculated based on the information obtained from other genes that HLA Class II introns would have even more variation than the exons because of the lack of selective pressure acting on them. Since there is only very little phylogenetic relationship between HLA Class I and Class II genes it is not possible to predict whether any structural features found in the Class I genes may also be present in Class II genes.

In the course of the present invention it has been surprisingly found that the histocompatibility locus antigen class II can be advantageously determined by using a group-specific primer or primer mixture located within intron 1 and another primer located at the 3'-end of exon 2 or in intron 2, preferably close to the 5'-end of intron 2, whereby this antisense primer should be highly conserved and preferably group-specific, in order to allow a high resolution typing of the HLA Class II genes.

The present invention relates to materials and methods for high-resolution, nucleic acid-based typing of the three classical HLA Class II antigens (DR, DQ, DP). It is based, in part, on the discovery of group-specific sequence motifs, derived from the analysis of numerous patient samples, which include sequences of intron 1. Such sequence motifs may be used to design amplification primers which may be used to identify the HLA group or type of a subject. The invention is also based, in part, on the determination of numerous allele-specific sequences which may be used to confirm the precise allelic type of a subject.

In the course of the present invention the remarkably conserved diversity of the HLA Class II introns which is lineage-specific rather than allele-specific has been discovered. The systematic serology-related diversity of HLA Class II intron sequences forms the basis of the present invention. This feature of HLA Class II introns has been proven to be extremely beneficial for setting up amplification strategies for template preparation.

It is a very important aspect of the present invention that by using a pair of primers several alleles can be amplified. The term "group" refers to such groups which can be distinguished by serological methods. In the present invention the groups DR1, DR2, DR3 and DR4-DR18 are of special importance. Usually in the serology of HLA groups so-called main antigens are to be distinguished from so-called split antigens. The later discovered split antigens carry group-specific epitopes and by using the split epitopes a main antigen can be subdivided in two split antigens. It should be noted that the main antigens are designated as DR1-DR10 whereby the split antigens are designated by DR11-DR18. The following relationship between the antigens should be mentioned:

DR5 can be subdivided in groups DR11 and DR12;
DR6 can be subdivided in groups DR13 and DR14;
DR2 can be subdivided in groups DR15 and DR16;
DR3 can be subdivided in groups DR17 and DR18.

For the main antigens DR1, DR4, DR7, DR8, DR9 and DR10 up to now no split antigens are known.

Each of the above-mentioned groups can be distinguished from each other by serological methods. With the help of molecular genetic methods, however, it is possible to identify a plurality of alleles for each group which have the same phenotype corresponding to the same serological appearance. The group DR11, for example, can be differentiated to presently about 30 alleles which show the same serological appearance.

Considered on the molecular level a DR antigen consists of two polypeptide chains the so-called α- and β-chain. The polymorphic form of the DR antigens is exclusively located in the β1 domain of the β-chain. This β1 domain is encoded by exon 2 of the in total 6 exons encoding the β-chain. The α-chain is not polymorph. The gene coding for the α-chain is the DRA gene and the β-chain is encoded by the gene DRB. The single genes coding for DRB have numbers and are presently designated as DRB1-DRB9. Since the genes DRB2, 6, 7, 8 and 9 are not expressed pseudogenes only the genes DRB1, DRB3, DRB4 and DRB5 are expressed. Each polypeptide chain of each of those DRB genes is associated with the same α-chain encoded by the DRA gene.

A heterodimer of polypeptide chains of DRA and DRB1 shows the serologically distinguishable specificities DR1-DR18. A heterodimer of the polypeptide chains of DRA and DRB3 shows the serologically distinguishable specificity DR52. A heterodimer of polypep-

tide chains DRA and DRB4 shows the serologically distinguishable specificity DR53 and a heterodimer consisting of the polypeptide chains of DRA and DRB5 shows the serologically distinguishable specificity DR51.

The antigens DR51, DR52 and DR53 are separate DR molecules on the surface of the cells. Those molecules are only present when certain DR1-DR18 molecules are also present. For example the antigen DR51 is only present if also DR15 or DR16 are present. DR52 is only present if DR3, 11, 12, 13, or 14 are present and DR53 is only present if DR4, 7 or 9 are also present.

By using the term "associated group" it is to be understood that for example group DR52 comprises also the antigens DR3, DR11, DR12, DR13 and DR14.

The sequence database of HLA-DRB genes is mainly derived from mRNA analysis or has been concentrated on the polymorphic second exon. In a preferred embodiment of the present invention the structural features of the intron sequences of the HLA-DRB 1, 3, 4 and 5 genes are used.

A sequence compilation of HLA-DRB 1, 3, 4 and 5 of the 3' 500 bp adjacent to the exon 2 of the 6400 bp first intron is disclosed.

The sequences used in the present invention were derived from well defined cell lines and PCR typed clinical samples. They represent all serologically defined groups and their most frequent subtypes. Beside multiple gene-specific sequence motifs, the HLA-DRB intron sequences exhibit a remarkably conserved diversity which is lineage-specific rather than allele-specific. The present invention allows therefore further insights into the genetic relationship between different alleles of HLA Class II genes and opens up the possibility for new PCR-based typing strategies using intron located amplification primers. The conserved intronic diversity will provide the comfort to establish typing systems which promise not to demand regular updates when new alleles are discovered.

The present invention provides substantially purified nucleic acids which are capable of selectively hybridizing with group specific sequence motifs in untranslated regions of HLA-D and especially the HLA-DRB1,3,4 and 5 genes. Such nucleic acids, which may be comprised in a kit, may be used, alone or in conjunction with exon-based primers, to determine the group specificity of said alleles contained in a patient sample and to identify the specific alleles present.

In particular embodiments, the present invention provides methods of ascertaining the HLA Class II type of a subject which comprise performing a first amplification reaction which identifies the group type of the subject, and a second amplification reaction which produces allele-specific nucleic acids for sequencing.

In the present invention the following definitions are used:

"Allele" means one of the alternative forms of the gene in question;

"Amplification" means the process of increasing the relative abundance of one or more specific genes or gene fragments in a reaction mixture with respect to the other genes. A method of amplification which is well known by those skilled in the art is the polymerase chain reaction (PCR) as described in United States Patents Nos. 4,683,194, 4,683,195 and 4,683,202, which are incorporated herein by reference. The PCR process involves the use of pairs of primers, one for each complementary strand of the duplex DNA (wherein the coding strand is referred to as the "sense strand" and its complementary strand is referred to as the "antisense strand"), that will hybridize at a site located near a region of interest in a gene. Chain extension polymerization (without a chain terminating nucleotide) is then carried out in repetitive cycles to increase the number of copies of the region of interest many times. The amplified oligonucleotides are then separated from the reaction mixture and used as the starting sample for the sequencing reaction. Gelfand et al. have described a thermostable enzyme, "*Taq* polymerase," derived from the organism *Thermus aquaticus*, which is useful in this amplification process (see United States Patent Nos. 5,352,600 and 5,079,352 which are incorporated herein by reference);

"Group" as used herein, refers to a subset of alleles of one locus, all of which share sequence features which distinguish them from other groups. For example, serological group reactivity (in a lymphocytotoxicity assay) is the conventional basis for nomenclature of HLA alleles. The first two digits of an allele refer to the serological group; for example, the designation DRB1*0101, DRB1*0102, DRB1*0103 all are members of the DR1 group. Further, typically the nomenclature refers to the serological split group;

Since only the DRB genes are polymorphic there are only here different alleles. Those alleles are named for example for the group DR11: DRB1*1101, 1102, 1103 ... up to presently DRB1*1130. The first two digits refer to the serologically distinguishable group which corresponds in most cases to the split antigen. The third and fourth digit describe the subtype which can, however, be distinguished only by molecular genetic methods and means the relevant allele. It should be mentioned that there are also a fifth, sixth and seventh digit whereby the fifth digit describes mutations in the exons which do not result in a change of the amino acid sequence (e.g. DRB1*11011 or DRB1*11012). The sixth and seventh digit of the name of the allele describe variations in the non-

coding regions of the DRB gene, namely the flanking regions at the 5'- and/or 3'-region and the introns.

"Group-specific sequence motif" means a generally short, 1-25 nucleotide ("nt") sequence of nucleic acid which is found only in one or a few groups. Where a motif is shared by several groups in one region of the HLA locus, group-specific sequence motifs in other regions of the locus may serve as group-distinguishing features. The motif may share one or more nucleotides with the consensus sequence for the region;

"Haplotype" means the allele present on one chromosome;

"Heterozygote" means the presence of at least two different alleles of a gene;

"Homozygote" means the presence of a single species of allele of a gene;

"Locus" means a gene, such as HLA-DRB1 or HLA-DRB3;

"Locus specific" means an event or thing associated with only one locus;

"Patient sample" means a sample collected from a patient in need of HLA typing which contains a sufficient amount and quality of nucleic acid (preferably DNA) for the performance of an amplification reaction. A nonlimiting example of a suitable source is peripheral blood lymphocytes, tissue (including cell cultures derived therefrom), mucosal scrapes, spleen and bone marrow;

"Primer" means a polynucleotide generally of 5-50, preferably 12-28, nucleotides length which can serve to initiate a chain extension reaction;

"Sequencing" or "DNA sequencing" means the determination of the order of nucleotides in at least a part of a gene. A well known method of sequencing is the "chain termination" method first described by Sanger et al., Proc. Nat'l Acad. Sci. (USA) 74(12): 5463-5467 (1977) (recently elaborated in EP-B1-655506, and Sequenase 2.0 product literature (Amersham Life Sciences, Cleveland) incorporated herein by reference). Basically, in this process, DNA to be sequenced is isolated, rendered single stranded, and placed into four vessels. In each vessel are the necessary components to replicate the DNA strand, which include a template-dependant DNA polymerase, a short primer molecule complementary to a known region of the DNA to be sequenced, and individual nucleotide triphosphates in a buffer conducive to hybridization between the primer and the DNA to be sequenced and chain extension of the hybridized primer. In addition, each vessel contains a small quantity of one type of optionally detectably labeled dideoxynucleotide triphosphate, *e.g.*, dideoxyadenosine triphosphate ("ddA"), dideoxyguanosine triphosphate ("ddG"), dideoxycytosine triphosphate ("ddC"), or dideoxythymidine triphosphate ("ddT"). In each vessel, each piece of the isolated DNA is hybridized with a primer. The primers are then extended, one base at a time to form a new nucleic acid polymer complementary to the isolated pieces of DNA. When a dideoxynucleotide is incorporated into the extending polymer, this terminates the polymer strand and prevents it from being further extended. Accordingly, in each vessel, a set of extended polymers of specific lengths are formed which are indicative of the positions of the nucleotide corresponding to the dideoxynucleic acid in that vessel. These sets of polymers are then evaluated using gel electrophoresis to determine the sequence.

"Specific hybridization" means hybridization of one strand of a nucleic acid to its complement.

"Target sequence" means the preferred site for specific hybridization of a primer; and

"Untranslated region" refers to a portion of an HLA locus which may be part of the mature RNA, but are not translated into protein. Examples of untranslated regions are the 5' and 3' flanking regions and intron sequences. For example, the 5' flanking region is neither transcribed nor translated, and intron sequences are transcribed but not translated.

DESCRIPTION OF THE FIGURES

FIGURE 1 is an illustration of the principle for an HLA Class II sequencing strategy. Group-specific primers are used for PCR amplification, and group-overlapping primers located in intron 1 near the 2nd exon and at the 3'-end of exon 2 or at the 5'-end of intron 2 are used for sequencing.

FIGURE 2 shows an alignment of the 486 bp of intron 1 of HLA-DRB1,3,4 and 5 genes upstream of exon 2 compared to the HLA-DRB consensus sequence. Numbers above the seqences refer to the nucleotide position within the non-coding region. Dashes indicate identity with the consensus, asterix indicate deletions in the sequence. The 5'-end of the second exon is marked as a grey area.

FIGURE 3 A shows the sequence and the localization of the PCR primers for the group-specific amplification of different gene loci, especially HLA-DRB 1, 3, 4 and 5. In Figure 3 B the primers for sequencing are

shown.

The names of the sequencing primers show for which group they can be used. For example, sequencing primer DR2 can be used for sequencing the DR2 group. The sequencing primer DR3, 5, 6, 8, 10, B4 can be used with the groups DR3, DR5 (DR11 and DR12), DR6 (DR13 and DR14), DR8, DR10 of the DRB1 gene and for DR53 (the polymorphic gene coding therefore is named DRB4).

The sequencing primer DRB3.2 is exclusively relevant for the gene DRB3 and has been designated DRB3.2 in order to distinguish this sequencing primer from the amplification primer DRB3.1.

FIGURE 4 shows the specificity of the primer mixtures for the production of templates. The selection of the mixture of primers for sequencing was done on the basis of a low discriminating pretyping. The designation of the mixture of primers was chosen according to the serologically defined groups DR1-10, DR12-14 and DR51-53.

The used primers show a clear group-specificity which relates to the serologically defined groups. This specificity is expressed in the name of the primer mix. The primer mix having the name 1, for example, can be used in order to amplify the alleles of the DRB1 gene which causes the serological specificity DR1.

A more complicated example is the primer mix having the name 5. This primer mix can be used in order to amplify alleles of the serological specifity DR5, namely the subgroup DR11. Moreover this primer mix amplifies, however, the subgroups DR3, DR13 and DR14.

The group-specificity is based on the specificity of the 5'-primer located in the first intron (for example DRB01-DRB05). All primer mixes contain the antisense primer at the 3'-end named DRBAS. This primer is located within a sequence at the end of exon 2 having no variability. This sequence is common for all DRB genes (DRB1, 3, 4 and 5). Alternatively a group-specific primer located in the 5'-end of intron 2 could be used.

Since all primer mixes cover the variability of the exon 2 it is possible to determine by sequencing of the obtained PCR products the exact allele of the DRB gene.

It has been found that at the end of intron 1 there is no universal sequence which allows the design of a sequence primer usable for all groups. This represents an essential difference with regard to the situation of HLA Class I. Therefore, group-specific sequencing primers have to be used which has, however, the advantage that the quality of sequencing is improved. The sequencing primer (as shown in Figure 3b) have names corresponding to the groups for which they are specific.

FIGURE 5 shows primer mixes which can be used for pretyping. The primers are located in exon 2.

FIGURE 6 shows the nucleotide sequences of the primers mentioned in Fig. 5.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to compositions and methods which may be used to efficiently and accurately determine the HLA Class II type of a patient sample.

The present invention is based, in part, on the determination of group-specific sequence motifs in certain regions, especially intron regions of HLA Class II loci. These motifs may be used to design oligonucleotides which may be used as group-specific primers in nucleic acid amplification reactions. The present invention is also based, in part, on the determination of the sequences of regions of a wide variety of alleles of HLA Class II loci; such sequences may be used to distinguish one allele from another. Sequences of intron 1 of HLA-DRB genes are provided herein, and are set forth in Figure 2.

In general, the methods of the invention may be described as follows. Comparison of nucleotide sequences of an HLA locus among members of an HLA Class II group, which lie in either untranslated or exon regions, may be used to identify groupspecific motif sequences. Identification of groups may be by establishing serological relationships or using phylogenetic information. Based on the group-specific motif sequences, oligonucleotide primers may be designed, synthesized, and used to amplify a portion of the HLA locus. Oligonucleotides used in this manner are referred to herein as "group-specific primers" and, in particular, as "group-specific untranslated region primers" or "group-specific exon region primers", as the case may be.

In preferred nonlimiting embodiments of the invention, the primers correspond to untranslated regions of the HLA Class II locus ("group-specific untranslated region primers"). Such primers may be used in pairs, wherein each member of the pair hybridizes to an untranslated region lying on either side of at least one exon. For example, but not by way of limitation, primer pairs may be oligonucleotide pairs which hybridize to group-specific motifs in the first and second introns. In another example the 5'-primer hybridizes to a group-specific motif in the 1st intron, whereas a gene-specific 3'-primer at the 3'-end of the second exon outside the variable region of this exon is used.

The group-specific primers may be used in several different methods according to the invention. In a first series of nonlimiting embodiments, the group-specific primers may be used in a diagnostic manner to identify which allelic groups are present in a patient sample. In a second series of nonlimiting embodiments, the group-specific primers may be used to amplify sufficient amounts of a particular allelic fragment which is then subjected to direct nucleotide sequencing using suitable sequencing primers.

According to the first series of embodiments, the present invention provides for a method of determining

the HLA Class II group type of a subject comprising

(i) combining a group-specific primer pair with a target DNA sample from the subject under conditions such that primer-based amplification of the target DNA may occur; and

(ii) determining whether a nucleic acid product is produced by the amplification; wherein the ability of a primer pair to produce a nucleic acid product is associated with a particular HLA group type. The group-specific primers may be group-specific exon region primers or group-specific untranslated region primers.

In related embodiments the present invention provides for a method of determining the HLA Class II group type of a subject comprising

(i) combining a plurality of group-specific exon region primer pairs with a target DNA sample from the subject under conditions such that primer-based amplification of the target DNA may occur;

(ii) determining the size of the nucleic acid products of the amplification; and

(iii) correlating the size of the product with the predicted size of a fragment associated with a particular HLA group type. The plurality of primers is referred to as an HLA "cocktail".

These first methods may be used to provide useful diagnostic information. For example, group type determination may serve as a first level of comparison for a histocompatibility analysis, even without identification of the specific allele(s) involved. For example, if a potential donor and host are being evaluated for tissue transplantation, and, if it is found that their group types do not match, no further comparison may be necessary. If, alternatively, their types do match, further analysis, for example by direct sequencing, may be desirable.

According to the second series of embodiments, the present invention provides for a method of determining the HLA Class II allelic type of a subject comprising

(i) combining a group-specific oligonucleotide primer pair with a target DNA sample from the subject under conditions such that primer-based amplification of the target DNA may occur;

(ii) collecting the nucleic acid product of the amplification; and

(iii) determining the nucleic acid sequence of the product.

The group-specific primer pair used may be determined based on the group type of the subject, as determined using the first method, described above. In preferred embodiments of the invention, group-specific untranslated region primers which span a region of the HLA locus containing allele-specific sequence may be utilized. If a subject is heterozygous, separate amplification reactions are performed for each group identified. Sequencing may be performed using group-overlapping sequencing primers which will operate for several groups.

A more detailed description of the invention follows. Most alleles of the classical HLA Class II gene loci (consisting essentially of HLA-DRB genes) can be distinguished on the basis of exon 2 only. In one non-limiting embodiment, a method of the invention takes advantage of this fact, and employs the strategy generally described above, using the example of HLA-DRB.

A genomic DNA sample is prepared from a patient sample according to well known techniques. Aliquots of the genomic DNA may then separately be reacted with a panel of groupspecific exon region primer pairs, wherein the successful amplification of a DNA fragment is associated with a particular group type.

Alternatively, part of the sample may be treated with a cocktail of group-specific exon region primer pairs. Each primer pair in the cocktail will amplify only selected allelic groups because at least one of them specifically hybridize to group specific intron sequence motifs. Between them, under suitable polymerase chain reaction (PCR) conditions, the cocktail may amplify all known HLA-DR or HLA-DRB groups, with each group specific amplification product having a different length. When reaction products are separated on an agarose gel the group(s) present in the patient sample may be identified by length.

Optionally, once the group specificity is determined, the direct sequence of alleles may be determined for precise allelic identification. A further part of the patient sample DNA may be treated under PCR conditions with a pair of primers that are specific for the previously determined group; preferably such primers are group-specific untranslated region primers, which span greater distances of the locus. If two groups were detected, then two separate reactions are performed. At completion of the second amplification, the reaction products are sequenced using an intron based "sequencing primer" which hybridizes to an intron sequence which may be conserved among alleles of several groups. Though it is theoretically possible to use a sequencing primer which is specific for the amplified group only, it is found that using a group-overlapping primer simplifies the method and the preparation of a kit. Various group-overlapping sequencing primers are specifically provided herein which hybridize, respectively, to intron sequences flanking the 5' end of exon 2.

The substantial advantage of the method of the invention is that the initial group specific amplification allows a PCR based separation of haplotypes in 95% of

patient samples. The separation of the haplotypes is a major achievement of this protocol since it permits the resolution of cis/trans linkages of heterozygote sequencing results which cannot be achieved with other protocols. With the instant invention, a separation of the haplotypes may be achieved in serological heterozygous samples with the sequencing primer mixes ("PMs") using group-specific amplification corresponding to the serological families. The selection of the PMs used for sequencing depends on the amplification patterns of the preceding PCR-SSP low-resolution typing. The primers are designed to work within a single cycle protocol including, but not limited to, a PCR protocol on a Perkin Elmer System 9600, maintaining typing capacities of the laboratory. All PCR products carry sufficient sequence information for a complete subtyping. This approach is superior to a typing system using a single pair of generic primers followed by direct sequencing or SSO hybridization, even if the amplification strategy is locus-specific. The substantial advantage of Sequencing Based Typing (SBT) is the definition of the cis/trans linkage of sequence motifs. SBT after generic PCR amplification cannot define the cis/trans linkage of sequence motifs and therefore mimics oligotyping. The rapidly growing number of newly identified alleles confirms that new alleles have arisen mainly from gene conversion events which have usually taken place between different alleles of the same locus. Newly identified alleles are not characterized by new sequence motifs, but by a new combination of already existing sequence motifs. From this observation it may be concluded that the amount of alleles at each locus may theoretically represent all possible combinations of known sequence motifs. Of course, some of them will fall victim to negative selection. Nevertheless, it can be expected that still an enormous amount of alleles are yet unidentified. PCR-SSP subtyping strategies using a restricted number of oligonucleotides which do not cover all possible sequence motifs suffer from this limitation. If the cis/trans linkage of the analyzed polymorphic regions is not defined some new alleles may be mistyped as a heterozygous combination of known alleles. This has consequences with respect to SBT strategies. An unambiguous typing result of SBT after generic PCR amplification is only unambiguous with regard to the presently known HLA sequence databank.

In general, group-specific primers are desirably designed to facilitate hybridization to their intended targets. It should be taken into account that homology between different groups, and indeed between group-specific motifs, may exist. Accordingly, in preferred embodiments of the invention, a primer may be designed such that it hybridizes to its group target under relatively stringent conditions. For example, one or more mismatched residues may be engineered into the 3' domain of the molecule. Further, the primer may be designed such that it differs from any naturally occurring or consensus sequence, but rather has mismatches inserted which serve to further reduce hybridization of the primer to target DNA of a group other than the intended target group. Under certain circumstances, one or more mismatches may be introduced into the 5' end to destabilize internal hairpin loops; such changes are not generally expected to enhance the efficiency of the primer.

The nucleic acid sequences as given in Figure 3 may be comprised in group-specific untranslated region primers for HLA-DRB, which are specific for the groups as indicated in Figure 4.

In general, the foregoing group-specific primers may be modified by addition, deletion, or substitution of bases, to produce functionally equivalent primers with the substantially same specificity, that is to say, such that the group specific polymorphism(s) are not removed. Such modifications may be constrained by several parameters. First, exact matching at the 3' end is particularly important for primer extension. Preferably, at least 5 nt are complementary to target DNA. When the exactly conserved region is short, for example, less than 10 nt, it is not advisable to change the primer sequences. The primer is preferably less than 50% G or C. Also, the primers should be designed to avoid specific hybridization with pseudogenes. In the examples which follow, the melting temperature of all primers used was about 62°C to ensure uniform amplification conditions.

In a further preferred embodiment of the present invention a pretyping is performed. In the course of this pretyping it is checked which groups are present in the patient. Because of the diploid chromosomes only two groups are possible. It would be possible to use all primer mixes in parallel assays with one probe and afterwards it is checked which primer mix has resulted in an amplification product. This product can then be sequenced. In one preferred embodiment a pretyping is done by using primer mixes located within the second exon. Those primer mixes amplify group-specific only a small fragment of exon 2. These primer mixes are, however, not suitable for sequencing since they contain only a small part of the variability of the exon and by sequencing no further information is obtained. The primers are shown in Figure 5. The sequences of the primers disclosed in Figure 5 are shown in Figure 6.

In this embodiment of the present invention a pretyping is done by using the primers located in the exon and in a second step the primer mix as shown in Figure 4 is used for the preparation of the template. This is possible since there is a strict correlation between the intronic sequences and exonic sequences.

The selection of suitable universal sequencing primers is constrained by a variety of rules including the following. Sequencing primer hybridization sites must lie within the fragment amplified by the group specific amplification primers. All primers are desirably selected to provide informative sequence and not start too far upstream of useful sequence. Preferred primers hybrid-

ize to conserved sites near the exon/intron boundaries.

An important issue of direct sequencing for HLA class II genes is the generation of a specific PCR product, which is rather complicated due to the extensive sequence homologies between the different HLA class II loci including several pseudo-genes. If an adequate PCR product has been generated, any sequencing chemistry should be applicable.

In the normal case, since group specific amplifications take place before sequencing, only one allele at a time is sequenced, resulting in unambiguous homozygous sequencing results. In these cases alleles are relatively easy to identify, even without software.

However, in about 5% of cases, both alleles come from the same group, but the sequence results show heterozygosity. In practice, when viewed by a fluorescence-detecting system, the sample appears as a normal sequence of bases with, sporadically, two bases at one site, each with half the peak height. This result flows from the high degree of similarity shared among all alleles of each HLA gene; sequence heterozygosity flows from base substitutions. The laborious task of determining which alleles are present in the test sequence may be simplified using computer analysis. A software program, for example GeneLibrarian (obtainable from Visible Genetics) rapidly compares the test sequence to a database which includes all possible homozygote and heterozygote combinations of the alleles. The program identifies those stored sequences that are closest matched to the test sequence. The operator can then determine which allelic pair is in the test sample. If no allelic pair shows an exact match, the software allows the operator to review the test sequence to determine if errors in base-calling or other artifacts are interfering with the analysis.

The order of sequencing reactions may be selected by the operator. Each exon of each locus may be sequenced on the sense strand or anti-sense strand. A preferred method is to obtain sequence from one strand from the exon. If the results contain ambiguities, then the amplicon is re-sequenced using the other primer for the same exon. The availability of both sequencing primers provides redundancy to ensure robust results.

In some cases, it may be advantageous to employ an equimolar mixture of 2 or more oligonucleotide species. Mixtures of oligonucleotides may be selected such that between them they will effectively prime the sequencing reactions for all alleles of the locus at the same site.

In an alternative technique, instead of using dye terminators, a dye-labelled primer may be employed. In this case, the selected sequencing primers is labelled on the 5' end with a detectable label, using phosphoramidite or NHS/dye ester techniques well known in the art. The label selected depends on the detection instrument employed. The label for use with an OpenGene System (Visible Genetics Inc., Toronto, ON) is the fluorophore Cy5.5 (Amersham Life Sciences, Cleveland OH). Fluorescein-isothio-cyanate may be used for detection with the ALF Automated Sequencer (Pharmacia, Piscataway NJ). In this method, which is well known to one skilled in the art, the sequencing reaction mixture is changed slightly to include only one ddNTP per reaction mixture. For detection of reaction products, the sample may be mixed with an equal volume of loading buffer (5% ficoll plus a coloured dye). 1.5 µl of these samples may be loaded per lane of a MicroGel electrophoresis cassette loaded in a MicroGene Blaster automated DNA sequencer (Visible Genetics Inc., Toronto). The sample may be electrophoresed and read.

Results may be displayed and analyzed with GeneObjects software. The sequence of bases may be determined, and the HLA allele to which the sequence corresponds may then be identified. This process may be performed for each locus (HLA-DRB1-5) and the results may then be reported to the patient file.

It is well known in the art that different variations of sequencing chemistry may be employed with different automated DNA sequencing instruments. Single dye instruments, such as the OpenGene System (Visible Genetics Inc., Toronto), the ALF Express (Pharmacia, Uppsala, Sweden) or the Li-Cor 4000L (Lincoln City, Nebraska) generally use dye-labeled primers. In these systems a single chain termination sequencing reaction mixture is run per lane.

Multi-dye sequencers, such as the Prism 377 (Applied Biosystems, Inc., Foster City, California) detect multiple dyes in a single lane. This technology conveniently employs dye-terminator chemistry, where the chain-terminating nucleotides are themselves labeled with fluorophores (see United States Patent No. 5,332,666, to Dupont de Nemours and Co.). In this case, the reaction products carrying four different labels may be run in a single lane.

Either single dye or multi-dye chemistry may be employed according to the present invention, along with other sequencing chemistries.

The nucleic acids described above may be comprised in a kit for use in practicing the methods of the invention. In addition to the group-specific primers and primer pairs disclosed, such kits may further comprise buffers, reagents, and enzymes such as, amplification enzymes including but not limited to, *Taq* polymerase. In specific, non-limiting embodiments, the kit may comprise group-specific exon region primers (for example, as a "cocktail" comprising a plurality of primers) as well as group-specific untranslated region primers; such primers may be contained in individual tubes.

Methods of high resolution typing are detailed in the examples below, which examples are set out to exemplify the method of the invention and not to limit the scope of it in any way.

HLA-DRB genes are about 12 KB in size. About 93% of the genes are made up by non-coding sequences, whereas only 801 bp from exon 1 through exon 6 carry the coding information, representing less

than 7% of the gene's nucleotides. The polymorphism of the coding region is exclusively restricted to the β1-domain encoding exon 2 which represents the preferred embodiment of the present invention.

Based on the diversity of this exon 2 216 HLA-DRB 1, 3, 4, and 5 alleles can be distinguished according to the latest WHO nomenclature report [Tissue Antigens (1997), vol. 49, p. 297-321]. This gives impressive insights in the peptide presenting capacity of the human population and it can be expected that a tremendous number of alleles will be identified in the future.

Despite profound knowledge of the different coding regions nearly nothing is known about the intervening intron sequences.

The HLA-DRB intron sequences are not characterized by random point mutations but by a highly systematic diversity, reflecting the different DRB lineages. With a few more exceptions than in HLA Class I the variability is arrested on the level of the serological diversity with almost no sequence variations between the subtypes of the same serological group.

The few intragroup variations detected in DRB1*15 (1502 and 1503), DRB1*13 (1302, 1303, 1305) and DRB1*08 (0803) as well as in DRB4 and 5 alleles are with the exceptions in DRB1*13 (nucleotides 54 and 370) and DRB4*0101 (nucleotide position 61) all unique point mutations, which cannot be explained by intronic or interlineage recombination events. These deviations do not affect the group-specific intron motifs. The differences found in DRB3 alleles are in accordance with the serology-related character of the intronic diversity, showing sequence differences between DR52a, 52b and 52c, but not between the two alleles belonging to DR52b (DRB3*0201 and 0202).

In the course of the present invention no evidence has been found that recombination events diversifying the exons do involve the intron regions. However, this cannot be completely excluded.

The striking conservation within each ancestral lineage suggests that point mutations have been negatively selected. Consequently it can be assumed that introns carry a functional relevance and that they are not as much subjected to evolutionary diversification forces as the exons. Based on the finding of the present invention it can be expected that the serology-related motifs in the non-coding regions are highly conserved, even if the intron sequences are not available from all alleles. Whatever the function is, it maintains the strictly exon-correlating variability of the intron sequences.

The finding of the present invention has substantial consequences for PCR based typing strategies. New alleles often cause ambiguous amplification patterns in exon-based PCR protocols and therefore demand a regular update of the typing system. This experience has been made in HLA Class I and II typing during the last years and will surely continue with the permanent detection of new alleles.

The intron-restricted amplification method of the present invention will probably not suffer from these limitations. Based on the now available intron data, it is assumed that new alleles will correspond to the intron sequence motifs described so far. Therefore, these motifs are selected as priming sites for group-specific PCR amplification.

Example 1

In the examples illustrating the present invention the following conditions were used:

a) DNA samples

Genomic DNA was prepared by a standard salting-out procedure. A set of 25 well defined lymphoblastoid B-cell lines mostly from the 9th and 10th international histocompatibility workshop and 191 PCR typed clinical samples, representing all serological HLA-DRB antigens from different ethnical backgrounds and their most frequent subtypes, were analysed.

b) PCR and sequencing primers

For sequencing of the non-coding regions, PCR-based template preparation was carried out, haplotype-specific by the use of a generic intron-located primer (19mer, 5' AgC ggA gTg gAg Agg TCT g 3' [SEQ ID No. 1], Tm 62°C) and group- or allele-specific primers in the 2nd exon. The design of the generic 5' primer relied on the very limited number of intron 1 sequences available from the EMBL databank. The resulting products carried, depending on the amplified allele, about 600 bp of the 1st intron of a single allele flanked by variable stretches of 2nd exon sequences. The PCR primers and several nested primers were applied as sequencing primers.

b) PCR conditions

The PCR reaction mixture in a final volume of 50 µl consisted of 500 ng genomic DNA, PCR-buffer [60 mM Tris-HCL, pH 9.5; 3.5 mM $MgCl_2$; 15 mM $(NH_4)SO_4$], 200 µM of each dATP, dCTP, dGTP and dTTP and 2.0 U DNA Taq Polymerase (Amplitaq™, Hoffmann-La Roche, Basel, Switzerland). For sequencing of the 1st intron biotinylated PCR products were generated using 10 pmol of the biotinylated generic 5' primer and 15 pmol of unlabeled 3' primers. PCR amplifications were carried out in a GeneAmp PCR System 9600 (Perkin Elmer Cetus Corp., Norwalk, CT). All primer mixes worked with the same PCR profile. After an initial denaturation step at 94°C for 2 min, samples were subjected to 15 two-temperature cycles, each consisting of denaturation at 94°C for 20 s, annealing and extension at 65°C for 50 s, followed by 20 three-temperature cycles with a decreased annealing temperature of 61°C and an extension at 72°C for

30 s. For visualization, 8 µl of the amplification product were run on a 2% agarose gel prestained with ethidium bromide (0.2 µg/ml).

d) Sequencing of PCR products

Sequencing of PCR products was performed using Amplitaq™ DNA Polymerase FS cycle sequencing chemistry and base specific fluorescence-labeled dideoxynucleotide termination reagents (dye terminators).

The PCR products were purified by streptavidin-coated paramagnetic Dynabeads M-280 Streptavidin (Dynal, Oslo, Norway). The biotinylated product was attached to the beads by incubating 40 µl of the PCR product for 15 min with 400 µg Dynabeads at 40°C. The beads had been washed previously and resuspended in 40 µl binding and washing buffer (B&W) (2 x buffer: 2 M NaCl, 10 mM Tris-HCL pH 7.5, 1 mM EDTA). After incubation, the PCR products were washed twice in B&W buffer and resuspended in 20 µl dH_2O. For the sequencing reaction, 1 µl of the purified PCR product was mixed with 3 pmol sequencing primer and 8 µl Terminator Ready Reaction Mix containing fluorescence labeled ddNTPs and alpha-thio dNTPs (Applied Biosystems, Foster City, CA). The final reaction volume was adjusted to 20 µl with dH_2O. Cycle sequencing was carried out in a GeneAmp PCR System 9600 for 25 cycles. After an initial denaturation step at 96°C for 10 s, the temperature was rapidly decreased to 50°C and held for 5 s, followed by the extension step at 60°C for 4 min. For the subsequent removal of dye-labeled nucleotides, Sephadex G-50 columns DNA Grade F (MicroSpin G50 columns, Pharmacia Biotech, Uppsala, Sweden) were used. The pellet was dried in a vacuum centrifuge and resuspended in 4 µl loading buffer containing deionized formamide/25 mM EDTA; pH 8.0 (5:1) and Blue dextran (50 mg/ml solution). Samples were heated for 2 min at 90°C to denature and 1.5 µl were loaded on a 0.2 mm thick 5% polyacrylamide-7M urea gel. Electrophoresis was run at constant 48 watt for 8 h on an ABI 377 automatic DNA sequencer (Applied Biosystems, software version 2.1.1).

Example 2

Alignment of the 1st intron sequences

All cell lines and clinical samples were sequenced for the investigated regions in both directions. Each of the serological defined groups was analysed several times in unrelated samples as indicated in Figure 1 (part I). Rare alleles could only be sequenced once or twice due to their limited availability. Figure 1 shows from the 6400 bp sized intron 1 the sequence alignment of the 486 bp 3' end fragment directly upstream of exon 2 and the deduced consensus of the coding HLA-DRB loci. Nucleotide agreement with the consensus is indicated by a hyphen (-), asterix (*) are introduced to achieve maximum alignment. The numbers above the sequences refer to the nucleotide positions and start at the 5' end of the sequenced fragment of the 6400 bp intron 1.

The intron sequences turned out to be highly polymorphic. Besides extensive homologies, numerous locus- and group-specific sites including stretches of nucleotide deletions could be identified. Most of the polymorphic motifs or deletions are related to serological families. This lineage specificity is the most remarkable feature of the intron sequences. Beyond that, there are close relationships between the HLA-DRB1 alleles belonging to the same haplotype group, i.e. the alleles of the DR51, DR52 and DR53 associated groups. The highest sequence similarity has been found in the DR51 associated alleles of DRB1*15 and 16. In the DR53 associated groups DRB1*0701 and DRB1*0901 are closer to each other than one of them to DRB1*04. The DR52 associated alleles exhibit strong homologies with the exception of DRB1*12, which exhibits numerous individual motifs. The DRB1*11 and DRB1*13 alleles are closer to each other than one of them to DRB1*14 alleles.

With two exceptions, for each serological group at least one unique sequence motif not shared by any other group has been identified. The DRB1*11 alleles do not exhibit any motif which is more specific than the common DR52 associated DRB1 group motifs. The individual motifs in DRB1*16 are shared with DRB1*04 alleles at position 141 and with DRB5 alleles at position 181.

The sequences turned out to have an extensive stability within each lineage. Individual sequence motifs of alleles belonging to the same serologically defined DRB1 group are the exceptions to the rule. Allelic single nucleotide differences have been found in the DRB1*15 group between positions 211-220. In the DRB1*13 group the DRB1*1302 bears the consensus sequence at position 54 and a unique motif at position 328, the DRB1*1302 and 1305 are lacking the DR13 specific motif at position 370. In the DRB1*08 group the DRB1*0803 has a unique motif at position 342.

The DRB3 alleles follow the same rule with complete intron sequence homologies within the serologically defined DR52a, b and c groups, but clearly unique differences between them. Within the DRB4 group some individual motifs could be identified in the DRB4*0101 allele (positions 2, 27, 61 and 412). Among the two DRB5 alleles sequenced so far (DRB5*0101 and 0202) for their 1st intron four differences could be identified (positions 49, 287, 305 and 438).

Figure 3 shows different primers which were perferably used for the amplification and sequencing of the specimens to be classified. Figure 4 shows a compilation of different primers and their use in determining the different HLA Class II genes or HLA-DRB genes.

Example 3

For typing the group of a patient in the first step the DNA is isolated from a blood sample. The isolated DNA is checked in the first step with the low-resolution typing system containing 24 primer mixes (as shown in Figure 5). This method can be designated as "PCR-SSP". The abbreviation "SSP" means sequence specific primer since the primer is specific for certain sequence motifs and only if those sequences are present in the DNA obtained from the patient an amplification of the DNA is obtained. The information obtained by this type of amplification is therefore simply obtained from the presence or absence of an amplification product which may be obtained by performing the different amplifications in parallel.

In the present example an amplification was obtained by using the primer mix 1.1, 15 and 24. This means that the patient shows the serology equivalent type HLA-DR1, 15; DR51.

In the next step the alleles of DRB1*01, DRB1*15 and DRB5* have to be determined. Therefore, suitable primer mixes as listed in Figure 4 are used in order to prepare products suitable for sequencing. In the present example the primer mixes number 1, 2 and 13 were used. After amplification the sequencing primers as given in Figure 4 were used.

By using this procedure the following type of the patient could be obtained: HLA-DRB1*0102,1501; DRB5*0202.

A typing as described in the present example has the advantage that the cis/trans linkage of the mutations in exon 2 can be defined. Many different alleles of the DRB gene are not characterized by specific sequence motifs but by a different compilation selected from a pool of sequence motifs. If the single alleles are not amplified in single batches but both alleles together (in the present example DRB1*0102 and 1501) different heterozygous positions are obtained by sequencing. From the results it cannot be concluded which positions are linked on one chromosome and which position belongs to the other chromosome. In these cases the typing is done by using suitable software which checks all possible cis/trans linkages against the most recent sequence databank. In case new alleles are discovered later it may happen that the type is no longer correct since the future sequence databank comprising further alleles may now also find further possible cis/trans linkages. This is a very common problem of known sequencing techniques and also the problem of the "PCR-SSO". The term "SSO" means hybridizing with sequence-specific oligonucleotides. In this method the PCR product obtained after a gene locus-specific amplification comprising usually at least two alleles is tested with many different oligonucleotides and on the basis of the so obtained sequence motifs and based on the sequence databank a typing is deduced.

Claims

1. A method of determining the HLA Class II group type of a subject comprising the following steps:

   (i) combining a group-specific primer pair with a target DNA sample from the subject under conditions such that primer-based amplification of the target DNA may occur; and

   (ii) determining whether a nucleic acid product is produced by the amplification;

   wherein the ability of the primer pair to produce a nucleic acid product is associated with a particular HLA group type.

2. The method of claim 1, wherein the HLA Class II group to be determined is part of the HLA-DRB locus.

3. The method of claim 1, further comprising the step of

   (iii) determining the nucleic acid sequence of the nucleic acid product of step (ii).

4. The method of claim 1, wherein the primer pair comprises one or more oligonucleotide primers selected from the group consisting of the primers of Figure 3.

5. A method of claim 1 wherein at least one primer is located in the untranslated region of the HLA Class II gene.

6. A method of determining the HLA Class II allele type of a subject comprising the following steps:

   (i) combining a group-specific exon region primer pair with a target DNA sample from the subject under conditions such that primer-based amplification of the target DNA may occur;

   (ii) determining whether a first nucleic acid product is produced by the amplification wherein the ability of the primer pair to produce a nucleic acid product is associated with a particular HLA group type, and thereby identifying the group type;

   (iii) combining a group-specific untranslated region primer pair corresponding to the group type of the subject, as determined by steps (i)-(ii), with a target DNA sample from the subject under conditions such that primer-based amplification of the target DNA may occur and a sec-

ond nucleic acid product is produced; and

(iv) determining the nucleic acid sequence of the second nucleic acid product collected in step (iii).

7. The method of claim 6, wherein the HLA Class II allele to be determined is part of the HLA-DRB locus.

8. The method of claim 6, wherein the group-specific exon region primer pair used in step (i) comprises one or more oligonucleotide primers selected from the group consisting of the primers of Figure 6.

9. The method of claim 6 wherein the group-specific untranslated region primer used in step (iii) comprises one or more oligonucleotide primers selected from the group consisting of the primers of Figure 3.

10. A method of determining the HLA Class II allele type of a subject comprising the following steps:

(i) combining a plurality of group-specific exon region primer pairs with a target DNA sample from the subject under conditions such that primer-based amplification of the target DNA may occur and a first nucleic acid product is produced;

(ii) determining the size of the first nucleic acid product of the amplification;

(iii) correlating the size of the first nucleic acid product with the predicted size of a fragment associated with a particular HLA type;

(iv) combining a group-specific untranslated region primer pair corresponding to the group type of the subject, as determined by steps (i)-(iii), with a target DNA sample from the subject under conditions such that primer-based amplification of the target DNA may occur and a second nucleic acid product is produced; and

(v) determining the nucleic acid sequence of the second nucleic acid product.

11. The method of claim 10, wherein the HLA Class II allele to be determined is part of the HLA-DRB locus.

12. The method of claim 10, wherein the plurality of groupspecific exon region primer pairs used in step (i) comprises one or more oligonucleotide primers selected from the group consisting of the primers of Figure 6.

13. A composition comprising a plurality of oligonucleotide primer pairs comprising one or more primers selected from the group consisting of the primers of Figure 3 and Figure 6.

14. A kit comprising:

(a) a plurality of oligonucleotide group-specific untranslated region primer pairs comprising one or more primers selected from the group consisting of the primers of Figure 3.

(b) an enzyme for nucleotide chain extension.

15. The kit of claim 14, further comprising:

(c) a cocktail comprising group-specific exon region primers comprising one or more primer selected from the group consisting of the primers of Figure 6.

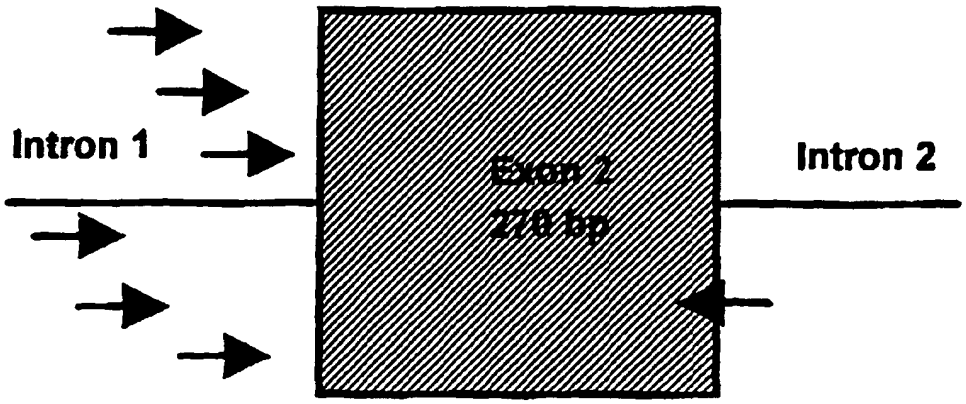

Fig. 1

Intron 1, part 1

```
                1        10        20        30        40        50        60        70        80        90       100
Consensus GTTTGGATTCCTAGAGGCAGCGCAGGCCAGGCACAAGGTCAGCACTAAGGAAGTGTTCACAGGATGAACGCGGTGGGTGCTGTTTAAGGAACCGGTAAAG
DRB1*0101 ----------C----------T----------G----------G----------G----------G----------G---------- (10)
DRB1*0102 ----------C----------T----------T----------G----------G----------G----------G---------- (2)
DRB1*0103 ----------C----------T----------T----------G----------G----------G----------G---------- (2)
DRB1*1501 ----------T----------T----------T----------G----------G----------G----------G---T------ (10)
DRB1*1502 ----------T----------T----------T----------G----------G----------G----------G---T------ (3)
DRB1*1503 ----------T----------T----------T----------G----------G----------G----------G---T------ (2)
DRB1*1601 ----------T----------T----------T----------G----------G----------G----------G---T------ (10)
DRB1*1602 ----------T----------T----------T----------G----------G----------G----------G---T------ (2)
DRB1*0301 ----------A----------T----------T----------G----------G----------A----------C---------- (10)*
DRB1*0401 ----------T----------T----------T----------G----------G----------A----------C----------AG------ (10)
DRB1*0402 ----------T----------T----------T----------G----------G----------A----------C----------AG------ (3)
DRB1*0403 ----------T----------T----------T----------G----------G----------A----------C----------AG------ (4)
DRB1*0404 ----------T----------T----------T----------G----------G----------A----------C----------AG------ (2)
DRB1*0405 ----------T----------T----------T----------G----------G----------A----------C----------AG------ (2)
DRB1*0407 ----------T----------T----------T----------G----------G----------A----------C----------AG------ (2)
DRB1*0408 ----------T----------T----------T----------G----------G----------A----------C----------AG------ (8)
DRB1*1101 ----------A----------T----------T----------G----------G----------G---------- (10)*
DRB1*1103 ----------A----------T----------T----------G----------G----------G---------- (2)*
DRB1*1104 ----------A----------T----------T----------G----------G----------G---------- (8)*
DRB1*1107 ----------A----------T----------T----------G----------G----------G---------- (2)*
DRB1*1201 ----------T----------T----------T----------G----------G----------G---------- (5)*
DRB1*1202 ----------T----------T----------T----------G----------G----------G---------- (2)*
DRB1*1301 ----------A----------T----------T----------G----------G----------G---------- (10)*
DRB1*1302 ----------A----------T----------T----------G----------G----------G---------- (4)*
DRB1*1303 ----------A----------T----------T----------G----------G----------G---------- (4)*
DRB1*1305 ----------A----------T----------T----------G----------G----------G---------- (3)*
DRB1*1401 ----------A----------T----------T----------G----------G----------G---------- (10)*
DRB1*1404 ----------A----------T----------T----------G----------G----------G---------- (2)*
DRB1*1408 ----------A----------T----------AA---------T----------G----------G----------G-AG------ (2)*
DRB1*0701 ----------A----------T----------T----------G----------G----------G----------G-AG------ (4)*
DRB1*0801 ----------A----------T----------A----------G----------G----------G---------- (8)*
DRB1*0802 ----------A----------T----------A----------G----------G----------G---------- (2)*
DRB1*0803 ----------A----------T----------A----------G----------G----------G---------- (3)*
DRB1*0804 ----------A----------T----------A----------G----------G----------G---------- (2)*
DRB1*0901 ----------A----------T----------AA---------T----------G----------G----------G-AG------ (4)*
DRB1*1001 ----------A----------T----------T----------T----------G----------A----------G---------- (3)*
DRB3*0101 ----------A----------T----------T----------T----------A----------A---------- (3)*
DRB3*0201 ----------A----------T----------A----------T----------A----------A---------- (3)*
DRB3*0202 ----------A----------T----------A----------T----------A----------A---------- (5)*
DRB3*0301 ----------A----------T----------A----------T----------A----------A---------- (5)*
DRB4*0101 -C----------G----------A----------T----------A----------G----------A----------G---------- (6)
DRB4*0102 ----------A----------A----------T----------A----------G----------G----------G---------- (6)
DRB4*0103 ----------A----------A----------T----------A----------G----------G----------G---------- (6)
DRB5*0101 ----------C----------T----------A----------T----------A----------A----------A-T-****-G----G------ (5)
DRB5*0202 ----------C----------T----------A----------T----------A----------G----------A-T-****-G----G------ (5)
```

Fig. 2 A

Intron 1, part II

Fig. 2 B

Intron 1, part III

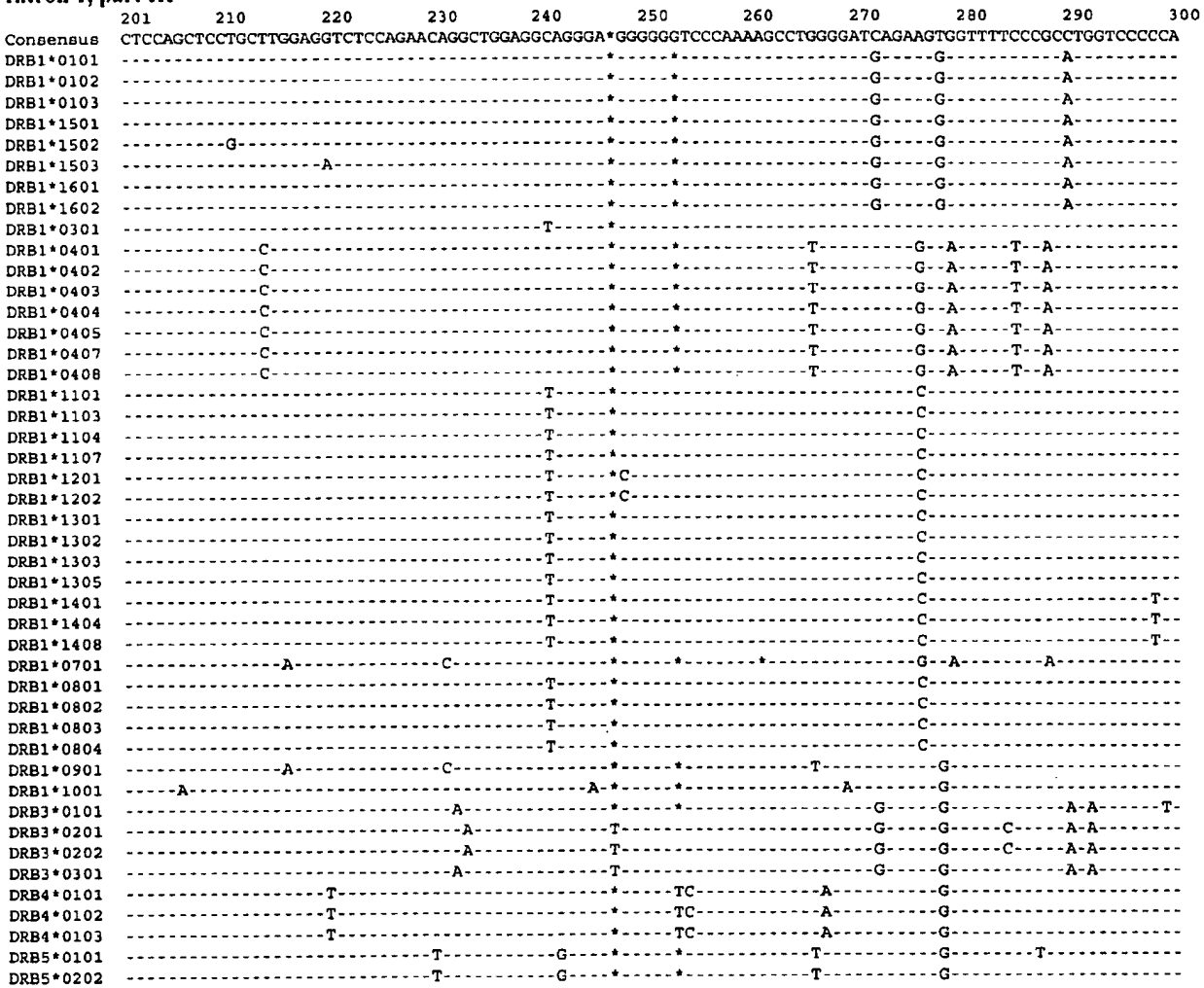

Fig. 2 C

Intron 1, part IV
301 310 320 330 340 350 360 370 380 390 400
Consensus GGC*CCCCGTTCGCCTCAGGAAGACAGAGGATGAGCCCCTGGCTGCAGGTGGTGGG*CGTTGCGGGTGGGGCCGGTTAAGGTTCCCAGTGCCCGCACCC
DRB1*0101
DRB1*0102
DRB1*0103
DRB1*1501
DRB1*1502
DRB1*1503
DRB1*1601
DRB1*1602
DRB1*0301
DRB1*0401
DRB1*0402
DRB1*0403
DRB1*0404
DRB1*0405
DRB1*0407
DRB1*0408
DRB1*1101
DRB1*1103
DRB1*1104
DRB1*1107
DRB1*1201
DRB1*1202
DRB1*1301
DRB1*1302
DRB1*1303
DRB1*1305
DRB1*1401
DRB1*1404
DRB1*1408
DRB1*0701
DRB1*0801
DRB1*0802
DRB1*0803
DRB1*0804
DRB1*0901
DRB1*1001
DRB3*0101
DRB3*0201
DRB3*0202
DRB3*0301
DRB4*0101
DRB4*0102
DRB4*0103
DRB5*0101
DRB5*0202

Fig. 2 D

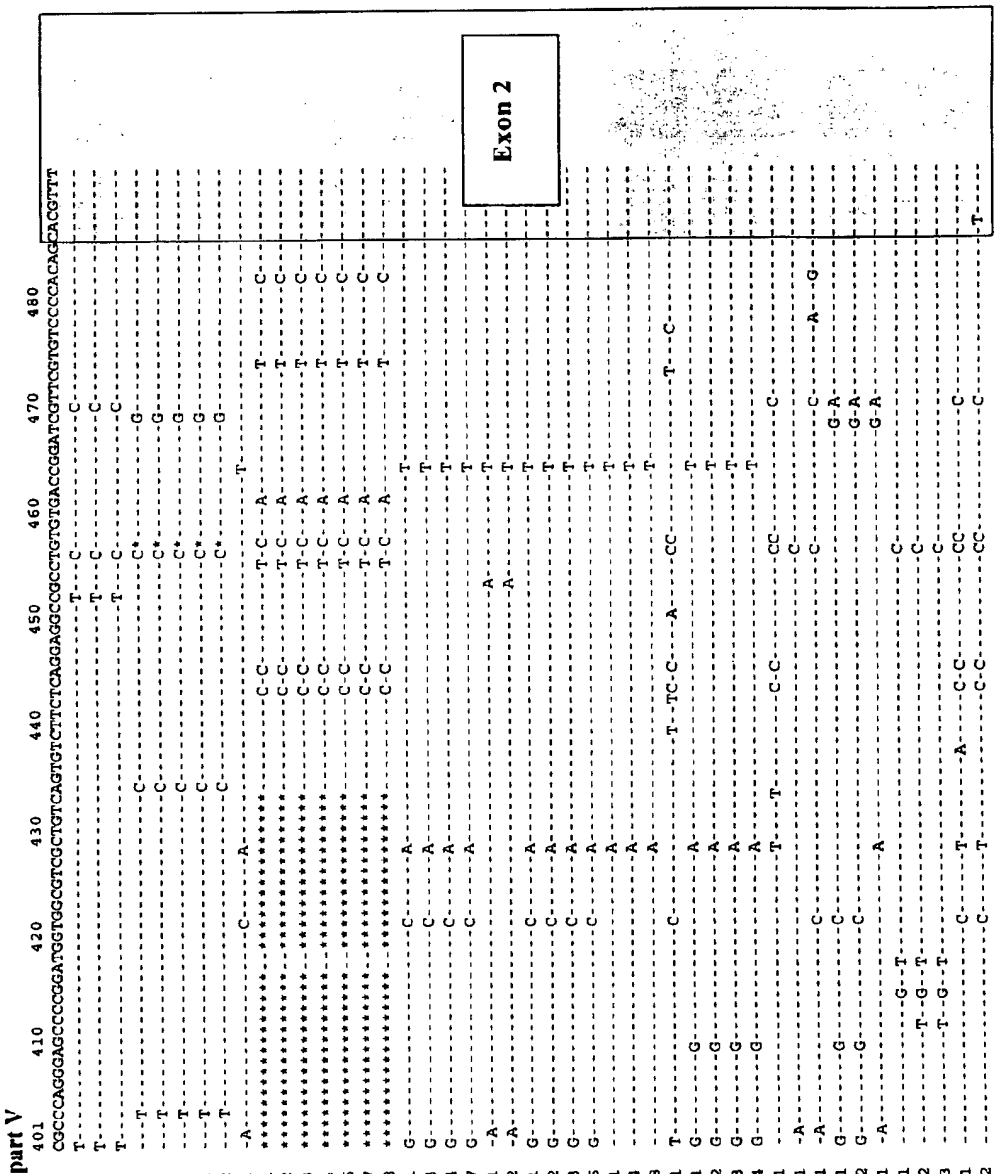

Fig. 2 E

amplification primer

Designation	Sequence	N	Tm°C	Position	Seq.ID No.
DRB01	S 5' TCC CAg gAg gAg gCg ggA 3'	18	62	167-174	2
DRB02	S 5' AgC gCC CgC ACC CCg CT 3'	17	62	388-404	3
DRB03	S 5' AAA AgC CTg ggg ATC AgA AgT 3'	21	62	258-278	4
DRB04	S 5' gCC CCT ggg CTg CgT gTT 3'	18	62	335-352	5
DRB05	S 5' Tgg Tgg gCg TTg Cgg Cg 3'	17	64	351-368	6
DRB12	S 5' AgT gTC TTC TCA ggA ggC CA 3'	20	62	435-454	7
DRB13	S 5' Tgg gCg TTT gCg gCg ggC 3'	17	60	354-370	8
DRB14	S 5' gAg CCC CTg ggC TgC AgA 3'	18	62	333-350	9
DRB07	S 5' gCg ggT ggg gCC ggg TC 3'	17	62	363-378	10
DRB08	S 5' AgC gCA ggC CAg gCA CAA A 3'	19	62	19-37	11
DRB09	S 5' ggA Tgg Tgg CgT CTC TgT T 3'	19	60	316-334	12
DRB10	S 5' ggC gTT gCg ggT ggg Cg 3'	17	62	366-374	13
DRB3.1	S 5' CCC CgT TCg CCT CAg gAA A 3'	19	62	305-323	14
DRB4	S 5' CCT CAg gAA gAC TgA ggA C 3'	19	62	314-332	15
DRB5	S 5' CCA gAA TAg gCT ggA ggC g 3'	19	62	224-242	16
DRBAS	AS 5' gCC gCT gCA CTg TgA AgC TC 3'	20	66	248-267	17

Fig. 3 A

sequencing primer

Designation	Sequence	N	Tm°C	Position	Seq.ID No.
DR1	S 5' gCC CgT gTg ACC ggA TCC 3'	18	62	454-471	18
DR2	S 5' CCg CCC TgT gAC Cgg ATg 3'	18	62	452-470	19
DR3,5,6,8,10,B4	S 5' ggA TCg TTC gTg TCC CCA C 3'	19	62	466-484	20
DR4	S 5' CCg gAg gCC gCT TCT gTA 3'	18	60	445-462	21
DR7,9,B5	S 5' CgC CCC TgT gAC Cgg ATC 3'	18	62	453-470	22
DRB3,2	S 5' gCT gTC AgT gTC TTC TCA gg 3'	20	62	429-448	23

Fig. 3 B

Primermix Nr.	Name	Sense Primer	Antisense Primer	HLA-DRB Spezifity	Sequencing Primer
1	1	DRB01	DRBAS	DRB1*0101-0104	DR1
2	2	DRB02	DRBAS	DRB1*1501-1506, 1601-1608	DR2
3	3	DRB03	DRBAS	DRB1*0301-0311	DR3,5,6,8 10,B4
4	4	DRB04	DRBAS	DRB1*0401-0424	DR4
5	5	DRB05	DRBAS	DRB1*0301-0311, 1101-1130 DRB1*1301-1330, 1401-1429	DR3,5,6,8 10,B4
6	12	DRB12	DRBAS	DRB1*1201-1205	DR3,5,6,8 10,B4
7	13	DRB13	DRBAS	DRB1*1301,1302 u.a.	DR3,5,6,8 10,B4
8	14	DRB14	DRBAS	DRB1*1401-1429	DR3,5,6,8 10,B4
9	7	DRB07	DRBAS	DRB1*0701	DR7,9,B5
10	8	DRB08	DRBAS	DRB1*0801-0816	DR3,5,6,8 10,B4
11	9	DRB09	DRBAS	DRB1*0901	DR7,9,B5
12	10	DRB10	DRBAS	DRB1*1001	DR3,5,6,8 10,B4
13	51	DRB5	DRBAS	DRB5*0101-0204	DR7,9,B5
14	52	DRB3.1	DRBAS	DRB3*0101-0301	DRB3.2
15	53	DRB4	DRBAS	DRB4*0101-0301	DR3,5,6,8 10,B4

Fig. 4

Fig. 5

HLA-DR "low-resolution" PCR-SSP primer mixes, version 1.0

primer mix no.	name	sense primer	antisense primer	size of product	HLA-DRB specifity
1	1.1	RB01	RB02	196 bp	B1*0101,0102,0104
2	1.2	RB01	RB03	197 bp	B1*0103
3	15	RB04	RB05	207 bp	B1*1501-1505
4	16	RB04	RB06/07	213,218 bp	B1*1601-1605
5	1317	RB08a	RB09	203 bp	B1*1317
6	3.1	RB10	RB45	130 bp	B1*0301,0304,0305, B1*0306
7	3.2	RB12	RB13	174 bp	B1*0302,0303
8	4	RB46a	BAMP-B	205 bp	B1*0401-0423
9	7	RB16	RB17	231 bp	B1*0701
10	8	RB08	RB07/18	165,216 bp	B1*0801-0814,1415
11	9	RB10	RB17	195 bp	B1*0901
12	10	RB60	RB20	268 bp	B1*1001
13	11	RB64	RB23	150 bp	B1*1101-1127,0415, B1*1411
14	12	RB24	RB25	183 bp	B1*1201-1204
15	13.1	RB49m 22	RB03/26/27	203,202 bp	B1*1301-04,1306, B1*1308-10,1312,1315, B1*1316,1319,1322, B1*1323 B1*1102,1114,1116, B1*1118-21,1416
16	13.2	RB21a	RB09/28/29	201,211 bp	B1*1301,1302,1304, B1*1307-08,1311, B1*1314-16,1318-24 B1*1101-04,1106, B1*1109-12,1114-16, B1*1120-22,1125,1416
17	14.1	RB49m 22	30am18 31am18	177 bp	B1*0301-0306,1301, B1*1316,1318-24, B1*1402,1403,1405, B1*1406,1409,1412, B1*1413,1414,1417-21, B1*1423,1424
18	14.2	RB08/ 21a	RB32	209,200 bp	B1*1401,1404,1405, B1*1407,1408,1411, B1*1414,1418,1113, B1*1117
19	14.3	RB33/ 34	RB35/36m6	146,145 bp	B1*1402,1406,1409, B1*1413,1417,1419, B1*1420,1421,1310
20	14.4	RB49m 22 RB37	RB38	224,150 bp	B1*1403,1412,1415, B1*1125,1318,0809
21	14.5	RB49m 22 RB14	RB39	173 bp	B1*1401,1407,1410, B1*1416,1422,1425

primer mix no.	name	sense primer	antisense primer	size of product	HLA-DRB specifity
22	52	RB40	RB41	241 bp	B3*0101,0201-0205, B3*0301
23	53	RB52	RB55	268 bp	B4*0101,0102-0104
24	51	RB44	RB06/27	147,164 bp	B5*0101-0105, B5*0201-0203

HLA-DRB Primer

Name	Sequenz	N	Tm	Orientierung	Seq.ID No.
DRB01S	5' CTT gTg gCA gCT TAA gTT TgA A 3'	22	62°C	sense	24
DRB04S	5' CCT gTg gCA gCC TAA gAg g 3' 1	9	62°C	sense	25
DRB08S	5' AgT ACT CTA Cgg gTg AgT gTT 3'	21	62°C	sense	26
DRB08aS	5' gAg TAC TCT ACg ggT gAg TgT T 3'	22	66°C	sense	27
DRB10S	5' gAC ggA gCg ggT gCg gTA 3'	18	62°C	sense	28
DRB12S	5' Cgg gTg Cgg TTC CTg gAg 3'	18	62°C	sense	29
DRB14S	5' gTT TCT Tgg AgC Agg TTA AAC A 3'	22	62°C	sense	30
DRB16S	5' ggT gCA gTT CCT ggA AAg ACT 3'	21	64°C	sense	31
DRB21aS	5' gTT TCT Tgg AgT ACT CTA CgT C 3'	22	64°C	sense	32
DRB24S	5' CAT AAC CAg gAg gAg CTC C 3'	19	60°C	sense	33
DRB33S	5' ggA CAg ATA CTT CCA TAA CCA g 3'	22	62°C	sense	34
DRB34S	5' ggA gAg ATA CTT CCA TAA CCA g 3'	22	62°C	sense	35
DRB37S	5' CTT CCA TAA CCA ggA ggA gTT 3'	21	62°C	sense	36
DRB40S	5' ACA gCA CgT TTC TTg gAg CT 3'	20	60°C	sense	37
DRB44S	5' gTg Cgg TTC CTg CAC AgA g 3'	19	62°C	sense	38
DRB46aS	5' gTT CCT ggA CAg ATA CTT CTA TC 3'	23	66°C	sense	39
DRB49m22S	5' ggT TTC TTg gAg TAC TCT ACg T 3'	22	64°C	sense	40
DRB52S	5' gAT CgT TCg TgT CCC CAC Ag 3'	20	64°C	sense	41
DRB60S	5' CAC AgC ACg TTT CTT ggA gg 3'	20	62°C	sense	42
DRB64S	5' ACg gAg Cgg gTg Cgg TTC 3'	18	62°C	sense	43
DRB02AS	5' CCg CCT CTg CTC CAg gAg 3'	18	62°C	antisense	44
DRB03AS	5' CCC gCT CgT CTT CCA ggA T 3'	19	62°C	antisense	45
DRB05AS	5' TCC ACC gCg gCC CgC gC 3'	17	64°C	antisense	46
DRB06AS	5' TAg gTg TCC ACC gCg gCg 3'	18	62°C	antisense	47
DRB07AS	5' TgC AgT Agg TgT CCA CCA g 3'	19	60°C	antisense	48

Fig. 6

Name	Sequenz	N	Tm	Orientierung	Seq.ID No.
DRB09AS	5' TCC ACC gCg gCC CgC TC 3'	17	62°C	antisense	49
DRB13AS	5' TCT gCA gTA gTT gTC CAC CC 3'	20	62°C	antisense	50
DRB17AS	5' ACC CCg TAg TTg TgT CTg CAC AC 3'	23	62°C	antisense	51
DRB18AS	5' CTg TTC CAg TAC TCg gCg CT 3'	20	64°C	antisense	52
DRB20AS	5' CTg CAC TgT gAA gCT CTC AC 3'	20	62°C	antisense	53
DRB23AS	5' CTg gCT gTT CCA gTA CTC CT 3'	20	62°C	antisense	54
DRB25AS	5' CTg CAC TgT gAA gCT CTC CA 5'	20	62°C	antisense	55
DRB26AS	5' CCC gCC TgT CTT CCA ggA T 3'	19	62°C	antisense	56
DRB27AS	5' CCg CgC CTg CTC CAg gAT 3'	18	62°C	antisense	57
DRB28AS	5' CCC gCC TgT CTT CCA ggA A 3'	19	62°C	antisense	58
DRB29AS	5' CCC gCT CgT CTT CCA ggA A 3'	19	62°C	antisense	59
DRB30am18AS	5' CTC gCT gTT CCA gTA CTC gg 3'	20	64°C	antisense	60
DRB31am18AS	5' TCT CgC TgT TCC AgT ACT CAg 3'	21	64°C	antisense	61
DRB32AS	5' CAC CTC ggC CCg CCT CC 3'	17	62°C	antisense	62
DRB35AS	5' CAC CgC ggC CCg CCT CT 3'	17	62°C	antisense	63
DRB36m6AS	5' gTC CAC CgC ggC gCg CTT 3'	18	64°C	antisense	64
DRB38AS	5' CTg CAg TAg gTg TCC ACC Ag 3'	20	64°C	antisense	65
DRB39AS	5' CTg TTC CAg TgC TCC gCA g 3'	19	62°C	antisense	66
DRB41AS	5' CgT AgT TgT gTC TgC AgT AAT 3'	21	60°C	antisense	67
DRB55AS	5' CTC CAC AAC CCC gTA gTT gTA 3'	21	64°C	antisense	68
DRBAMP-BAS	5' CCg CTg CAC TgT gAA gCT CT 3'	20	64°C	antisense	69

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 97 11 0438

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	WO 92 15711 A (UNIV MINNESOTA) see esp. p.28,l. 32 -p.30, claims and figure 1c	1-3,5-7, 10,11	C12Q1/68
X	EP 0 443 748 A (UNIV SINGAPORE) * the whole document *	1-3	
X	PETERSDORF E. W. ET AL.,: "Polymorphism of HLA-DRw52-associated DRB1 genes as defined by sequence-specific oligonucleotide probe hybridization and sequencing" TISSUE ANTIGENS, vol. 38, no. 4, - October 1991 pages 169-177, XP002047699 * the whole document *	1-3	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

C12Q

~~The present search report has been drawn up for all claims~~

Place of search	Date of completion of the search	Examiner
THE HAGUE	21 November 1997	Müller, F

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

European Patent Office

Application Number
EP 97 11 0438

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):

☐ No claims fees have been paid within the prescibed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

☐ All further search fees have been paid within the fixed time limit. The present Eurpean search report has been drawn up for all claims.

☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:

☒ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:

1-3,5,6,7,10,11 (completely) 4,9,13,14 (partly)

European Patent Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 97 11 0438

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1-3,5,6,7,10,11 (complete); 4,9,13,14 (partly)

   Method for determination of HLA Class II group in general as well as using primer Seq ID 2 and Seq ID 17, compositions and kits therefore.

2. Claims: 4,9,13,14 (partly)

   Method for determination of HLA class II group using primers Seq ID 3, compositions and kits therefore.

3. Claims: 4,9,13,14 (partly)

   Method for determination of HLA class II group using primers Seq ID 4, compositions and kits therefore.

4. Claims: 4,9,13,14 (partly)

   Method for determination of HLA class II group using primers Seq ID 5, compositions and kits therefore.

5. Claims: 4,9,13,14 (partly)

   Method for determination of HLA class II group using primers Seq ID 6, compositions and kits therefore.

6. Claims: 4,9,13,14 (partly)

   Method for determination of HLA class II group using primers Seq ID 7, compositions and kits therefore.

7. Claims: 4,9,13,14 (partly)

   Method for determination of HLA class II group using primers Seq ID 8, compositions and kits therefore.

8. Claims: 4,9,13,14 (partly)

   Method for determination of HLA class II group using primers Seq ID 9, compositions and kits therefore.

9. Claims: 4,9,13,14 (partly)

European Patent Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 97 11 0438

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

Method for determination of HLA class II group using primers Seq ID 10, compositions and kits therefore.

10. Claims: 4,9,13,14 (partly)

Method for determination of HLA class II group using primers Seq ID 11, compositions and kits therefore.

11. Claims: 4,9,13,14 (partly)

Method for determination of HLA class II group using primers Seq ID 12, compositions and kits therefore.

12. Claims: 4,9,13,14 (partly)

Method for determination of HLA class II group using primers Seq ID 13, compositions and kits therefore.

13. Claims: 4,9,13,14 (partly)

Method for determination of HLA class II group using primers Seq ID 14, compositions and kits therefore.

14. Claims: 4,9,13,14 (partly)

Method for determination of HLA class II group using primers Seq ID 15, compositions and kits therefore.

15. Claims: 4,9,13,14 (partly)

Method for determination of HLA class II group using primers Seq ID 16, compositions and kits therefore.

16. Claims: 4,9,13,14 (partly)

Method for determination of HLA class II group using primers Seq ID 18, compositions and kits therefore.

17. Claims: 4,9,13,14 (partly)

Method for determination of HLA class II group using primers Seq ID 19, compositions and kits therefore.

European Patent Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 97 11 0438

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

18. Claims: 4,9,13,14 (partly)

Method for determination of HLA class II group using primers Seq ID 20, compositions and kits therefore.

19. Claims: 4,9,13,14 (partly)

Method for determination of HLA class II group using primers Seq ID 21, compositions and kits therefore.

20. Claims: 4,9,13,14 (partly)

Method for determination of HLA class II group using primers Seq ID 22, compositions and kits therefore.

21. Claims: 4,9,13,14 (partly)

Method for determination of HLA class II group using primers Seq ID 23, compositions and kits therefore.

22. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 24, compositions and kits therefore.

23. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 25, compositions and kits therefore.

24. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 26, compositions and kits therefore.

25. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 27, compositions and kits therefore.

26. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 28, compositions and kits therefore.

European Patent Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 97 11 0438

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

27. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 29, compositions and kits therefore.

28. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 30, compositions and kits therefore.

29. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 31, compositions and kits therefore.

30. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 32, compositions and kits therefore.

31. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 33, compositions and kits therefore.

32. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 34, compositions and kits therefore.

33. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 35, compositions and kits therefore.

34. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 36, compositions and kits therefore.

35. Claims: 8,12,13,15 (partly)

European Patent Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 97 11 0438

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

Method for determination of HLA class II group using primers Seq ID 37, compositions and kits therefore.

36. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 38, compositions and kits therefore.

37. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 39, compositions and kits therefore.

38. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 40, compositions and kits therefore.

39. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 41, compositions and kits therefore.

40. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 42, compositions and kits therefore.

41. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 43, compositions and kits therefore.

42. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 44, compositions and kits therefore.

43. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 45, compositions and kits therefore.

European Patent Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 97 11 0438

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

44. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 46, compositions and kits therefore.

45. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 47, compositions and kits therefore.

46. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 48, compositions and kits therefore.

47. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 49, compositions and kits therefore.

48. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 50, compositions and kits therefore.

49. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 51, compositions and kits therefore.

50. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 52, compositions and kits therefore.

51. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 53, compositions and kits therefore.

52. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 54, compositions and kits therefore.

European Patent Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 97 11 0438

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

53. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 55, compositions and kits therefore.

54. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 56, compositions and kits therefore.

55. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 57, compositions and kits therefore.

56. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 58, compositions and kits therefore.

57. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 59, compositions and kits therefore.

58. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 60, compositions and kits therefore.

59. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 61, compositions and kits therefore.

60. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 62, compositions and kits therefore.

61. Claims: 8,12,13,15 (partly)

European Patent Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 97 11 0438

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

Method for determination of HLA class II group using primers Seq ID 63, compositions and kits therefore.

62. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 64, compositions and kits therefore.

63. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 65, compositions and kits therefore.

64. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 66, compositions and kits therefore.

65. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 67, compositions and kits therefore.

66. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 68, compositions and kits therefore.

67. Claims: 8,12,13,15 (partly)

Method for determination of HLA class II group using primers Seq ID 69, compositions and kits therefore.